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Expression Levels of the *CA9*, *WT1*, and *PRAME* Genes and Genotyping-Associated Antigens for the Diagnosis and Prognosis of Colorectal Cancer

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Abstract

Background Colorectal cancer (CRC) is the third most prevalent type of cancer worldwide, and is one of the major health problems in Asia, Africa, Europe, and America. The tumor antigens recently are of interesting indicators as diagnostic and prognostic tools, The aim of the present study is to detect the expression levels of *carbonic anhydrase IX* (*CA9*), the *Wilms tumor* gene (*WT1*), and the *preferentially expressed antigen in melanoma* (*PRAME*) in the peripheral blood of CRC patients in comparison with healthy controls.

Methods A prospective case-control study of CRC patients was conducted. We included 25 newly-diagnosed CRC eligible patients and obtained peripheral blood samples of them as well as 10 blood samples from the control group. All samples were then submitted to deoxyribonucleic acid (DNA) extraction and a molecular study through real-time polymerase chain reaction (PCR).

Results The CRC group consisted of 15 (60%) female and 10 (40%) male patients with a mean age of 50.52 ± 9.8 years, while the control group included 4 (40%) female and 6 (60%) male patients with a mean age of 47.7 ± 7.9 years. The CRC group, 24 (96%) of patient samples were *CA9*-positive with strong statistically significant differences ($p < 0.00001$; sensitivity: 96%; specificity: 90%). Regarding the *WT1* gene, there were 11 (44%) positive samples in the CRC group, with no statistically significant differences ($p = 0.055$; sensitivity: 44%; specificity: 90%). The *PRAME* gene was positive in 9 (36%) samples in the CRC group, with no statistically significant differences ($p = 0.357$; sensitivity: 36%; specificity: 80%. Among *CA9* (24 patients; 96%) of patients with CRC expressed positive results, in *WT1* 11(91.6%) CRC patients expressed gene, and in *PRAME* gene, 9 patients with CRC (81.8%) expressed positive results.

Conclusion Overexpression of the *CA9* gene in CRC of high sensitivity and specificity to be used as a tool to discriminate CRC from benign associate with high accuracy compare to WT1 and PRAME genes.

Keywords
- colorectal cancer
- tumor-associated antigens
- real-time polymerase chain reaction
- CA9
- WT1
- PRAME

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Introduction

In 2018, more than 2 million new cases of colorectal cancer (CRC) were recorded worldwide, and caused around 1 million deaths that year.[1–4] This type of cancer ranks third in terms of incidence and second in terms of mortality.[1] Tumor-associated antigens (TAAs) are molecules and proteins expressed by tumor cells, and they may act as cancer markers.[2] In cases of CRC, TAAs are underexpressed in normal tissue but are overexpressed or mutated in tumor cells.

A member of the carbonic anhydrase family, *carbonic anhydrase IX* (*CA9*) is one of the genes that is overexpressed in tumor cells; it is is a membrane protein that regulates cell proliferation in response to hypoxia.[5,6] In 2006, Talvinen et al.[7] found that *CA9* was the most up-regulated gene in cases of CRC through a complementary DNA (cDNA) microarray.

The *Wilms tumor* (*WT1*) gene is one of the genes involved in growth regulation and/or differentiation of cells.[8,9] Previous studies have demonstrated that *WT1* is expressed in cancer cells derived from different types of cancers, including CRC,[10] leukemia,[11] breast cancer,[12,13] non-small-cell lung carcinoma (NSCLC),[14] soft-tissue sarcoma (STS),[15] and head and neck squamous cell carcinoma (HNSCC).[16]

The *preferentially expressed antigen in melanoma* (*PRAME*) gene is detected in melanoma, but its expression is absent or low in cases of CRC.[17] The *PRAME* gene is expressed in many different cancers, and its expression correlates with prognosis and survival. It is expressed in NSCLCs, breast carcinomas, renal cell carcinomas (RCCs), head and neck cancers, Hodgkin lymphomas (HLs), sarcomas, Wilms tumors, and medulloblastomas.[17]

The present work was designed to assess the diagnostic and prognostic value of the *CA9*, *WT1*, and *PRAME* genes in cases of CRC.

Methods

Study Design and Setting

A prospective case-control study with CRC patients was conducted in the National Center of Cancer from March to December 2020. The following data were collected: age, gender, family history, tumor sites, tumor differentiation, and tumor stages. All patients were histologically diagnosed with CRC. The control group who referred to this center for screening purposes due to negative colonoscopies. Then all cases were submitted to molecular analysis.

Clinical Samples

Peripheral blood samples were collected from the 25 eligible patients newly-diagnosed with CRC in different stages, as well as from 10 patients in the control group. The samples had been sent to and preserved at the genetic laboratory of the National Center of Cancer. In total, 2 mL from each sample were put in ethylenediaminetetraacetic acid (EDTA) tubes. All samples were then submitted to DNA extraction and a molecular study through real-time polymerase chain reaction (PCR).

Ethics Consideration

All patients signed the informed consent form for the present study, and approval was obtained from the Ethics in Research Committee of Dijlah University College.

DNA Extraction and Real-time PCR

The total genomic DNA of the samples from the CRC and control groups was extracted from the peripheral blood using the salting-out method,[18] following the protocol provided by the manufacturer. Beta-catanin/Tcf is the constitutive gene, a double-stranded 15-nucleotide oligomer composed from first forward set contained *CCCT T TGATCT TACC*, and the second reverse set *CCCT T TGGCCT TACC*.[19]

These were analyzed using specific primers, which were designed using the Gene Runner software, version 4.0.9.68 Beta (►**Table 1**). The quantitative real-time PCR assay was performed in duplicate using the Applied Biosystems PCR Systems (Thermo Fisher Scientific, Waltham, MA, US). Synthesis of the suitable size PCR products were visualized by agarose gel electrophoresis. Genotyping was performed using the PCR-restriction fragment length polymorphism (PCR-RFLP) analytical method. The real-time PCR program is described and illustrated in ►**Table 2** and ►**Fig. 1** respectively.

Table 1 Sequences of the primers used in the present study

Primer		Sequence	Size (base pairs)
CA9	Forward	*5′-GTGGAAGGCCACCGTTTC-3′*	1,546
	Reversed	*5′-CTCGTCAACTCTGGCAAAGG-3′*	
WT1	Forward	*5′-AGGCTTTGCTGCTGAGGAC-3′*	1,962
	Reversed	*5′-CAGGTCATGCATTCAAGCTG-3′*	
PRAME	Forward	*5′-CTTTCCTCGAAGGCCACCT-3′*	1,479
	Reversed	*5′-GTTATTGTGAGGACCTTTAACGA-3′*	

Table 2 Real-time polymerase chain reaction program

No	Steps	Temperature (C°)	Time	Cycles
I	Initial denaturation	50	2 minutes	1
II	Denaturation	95	10 minutes	1
III	Annealing	95	15 seconds	30
		65	60 seconds	
		72	60 seconds	
IV	Final extension	95	15 seconds	1

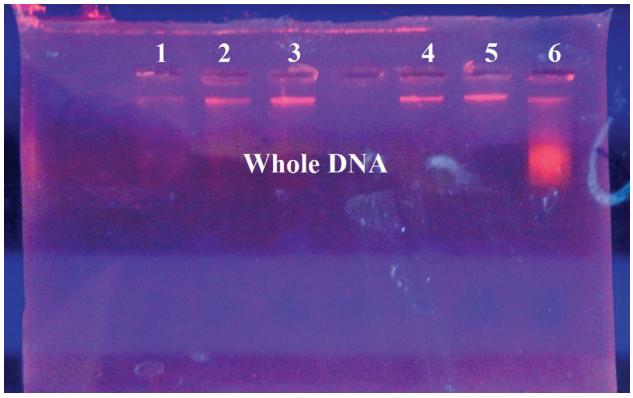

Fig. 1 Electrophoresis for the extraction of genomic DNA from samples in 1% agarose gel.

Statistical Methods

All data were analyzed using the Statistical Package for the Social Sciences (SPSS Statistics for Windows, IBM Corp., Armonk, NY, US) software. Frequency tables were analyzed using the Wilcoxon rank sum test to compare the two groups in a non-parametric pattern. The numerical variables were expressed as means and standard deviations (SDs). A two-sided p-value ≤ 0.05 was considered statistically significant.

Results

The CRC group consisted of 15 (60%) females and 10(40%) male patients with mean age of 50.52 ± 9.8 years, while the control group included 4 (40%) female and 6 (60%) male patients with mean age of 47.7 ± 7.9 years. Only 2 (8%) patients in the CRC group and 1 (10%) individual in the control group had family history positive of CRC. The rectosigmoid was the most common site, reported in 13 (52%) patients, the rectum in 10 (40%) patients, and the anus and rectum in 2 (8%) individuals. Regarding tumor differentiation, it was classified as moderate (52%), well (44%), and poor (4%). Regarding the tumor, lymph node, and metastasis (TNM) staging, T2N0, and T3N0 were the most common classes, each observed in 6 (24%) patients. Metastasis were found in 4 (16%) patients, and lymphovascular and regional invasions were observed in 12 (48%) patients. Stage-II cancer was the most common, present in 12 (48%) patients, as shown in ►**Table 3**.

Out of 25 cases of CRC, 24 (96%) patients samples were *CA9* positive, with strong statistically significant differences ($p < 0.00001$; sensitivity: 96%; specificity: 90%; positive predictive value [PPV]: 96%; negative predictive value [NPV]: 90%). Regarding the *WT1* gene, there were 44% of positive samples, with no statistically significant differences ($p = 0.055$; sensitivity: 44%; specificity: 90%; PPV: 91.67%; NPV: 39.13%). The *PRAME* gene was positive in 9 (36%) CRC patients, with no statistically significant differences ($p = 0.357$; sensitivity: 36%; specificity: 80%; PPV: 81.82%; NPV: 33.33%). As a result, the sensitivity and specificity of the *CA9* gene were higher for the diagnosis and prognosis of CRC than the *WT1* and *PRAME* genes, with an accuracy reaching 94.29%, as shown in ►**Table 4**. ►**Table 5** shows a comparison of the expression of TAAs of the *CA9*, *WT1*, and *PRAME* genes between the two study groups. Regarding the *CA9* gene, 24 (96%) patients in the CRC group and 1 (4%) individual in the control group expressed positive results, with strong statistically significant differences ($p < 0.0001$). s for the *WT1* gene, 11 (91.6%) CRC patients and 1 (8.4%) control expressed it, with strong statistically significant differences ($p < 0.0001$). In addition, 9 (81.8%) patients in the CRC group and 2 (18.2%) controls expressed the *PRAME* gene, with high statistically significant differences ($p < 0.001$). Gel electrophoresis of the PCR products of CRC patients were performed using the primers set for the *CA9*, *WT1*, and *PRAME* genes (forward and reversed) of DNA, as shown in ►**Fig. 2**.

Discussion

The incidence of CRC is increasing annually worldwide. The demographics of the CRC group were similar to those of the patients in most of the studies conducted in Iraq, such as those by Radhi et al.[20] in Al-Diwaniyah, by Alsafi et al.[21] in Karbala, by Alshewered and Al-Naqqash[2] and by Alrubaia et al.[3] in Baghdad, but differed from the demographics found in the studies by Alhilfi et al.[4] in Misan, and by Khalil et al.[22] in Duhok. In Western countries, 8% of the CRC cases are recorded in patients younger than 40 years of age, whereas in Asia and Africa the rates range between 15% and 35% for the same age group.[23–25]

Early detection of CRC can improve the chance of a successful management and better prognosis. Tumor-associated antigens and autoantibodies have generated increasing interest as biomarkers for the development of innovative diagnostic and prognostic tools that have an effective role in the management of human cancers.[26] Feitosa et al.[27] concluded that genetic counseling and proper screening programs are essential tools for the early diagnosis and follow-up of cases of CRC.

In the present study, *CA9* expression showed high sensitivity and specificity for the detection of CRC, with high accuracy (of up to 94.29%). In fact, it can be used as a potential significant prognostic tool in CRC. These data implicate that the intensity of the *CA9* expression seems to be a powerful independent prognostic factor, not confounded by the mode of treatment.

In a study, Korkeila et al.[29] found no correlation between the expression of CA9 and other clinical or histopathological factors of CRC, since most of their patients had received radiotherapy with or without chemotherapy before operation, which may have had an influence on tumor size, stage, grade, and nodal status. Driessen et al.[30] found a significant correlation between the *WT1* gene and the grade, stage, and lymph node status of different kinds of tumors, while Epping and Bernards[17] demonstrated that the lymph node status of patients was not directly associated with the *PRAME* gene.

In their study, Oji et al.[31] demonstrated that the *WT*1 gene was overexpressed in the majority of the primary colorectal adenocarcinomas they examined, and it was the non-mutated, wild type. This result supported their hypothesis that the wild-type *WT1* gene does play an oncogenic role in the

Table 3 Clinical features of patients with colorectal adenocarcinoma (colorrectal cancer group: patients 1-25; control group: patients 26-35)

ID	Age (years)	Gender	Family history	Tumor site	Differentiation	Tumor, Node, Metastasis classification	Stage
1	36	Female	Negative	Rectosigmoid	Well	T2N0	II
2	54	Female	Negative	Rectosigmoid	Moderate	T2N0	II
3	51	Male	Negative	Rectosigmoid	Well	T2N2	III
4	44	Female	Negative	Rectum	Moderate	T1N0	I
5	60	Male	Negative	Rectum	Well	T3N1M1	IV
6	37	Male	Negative	Rectosigmoid	Moderate	T3N0	II
7	29	Male	Negative	Rectosigmoid	Moderate	T3N1	III
8	53	Male	Negative	Rectum	Well	T2N0	II
9	63	Female	Negative	Rectum	Poor	T2N2M1	IV
10	56	Female	Negative	Rectum	Well	T3N0	II
11	39	Female	Positive	Rectosigmoid	Well	T3N1	III
12	55	Female	Negative	Rectum	Moderate	T3N0	II
13	40	Male	Negative	Rectosigmoid	Well	T2N2	III
14	54	Female	Negative	Rectosigmoid	Moderate	T2N0	II
15	52	Female	Negative	Rectosigmoid	Moderate	T3N1	III
16	70	Male	Negative	Rectosigmoid	Well	T3N0	II
17	62	Female	Negative	Rectosigmoid	Moderate	T2N1M1	IV
18	61	Female	Negative	Anus and rectumal	Moderate	T2N0	II
19	55	Male	Negative	Rectum	Moderate	T2N0	II
20	59	Male	Negative	Rectosigmoid	Moderate	T3N2M1	IV
21	48	Female	Negative	Anus and rectum	Moderate	T3N0	II
22	47	Male	Positive	Rectosigmoid	Moderate	T2N1	III
23	47	Female	Negative	Rectum	Well	T1N1	III
24	50	Female	Negative	Rectum	Well	T3N0	II
25	41	Female	Negative	Rectum	Well	T3N1	III
26	39	Male	Negative				
27	50	Male	Negative				
28	44	Male	Negative				
29	43	Female	Negative				
30	60	Male	Negative				
31	52	Female	Negative				
32	56	Female	Negative				
33	55	Male	Negative				
34	38	Female	Positive				
35	40	Male	Negative				

tumorigenesis of CRC. The results of the present study are in line with those of the study by Oji et al.[31] since the *WT1* was expressed in 44% of the CRC group, but without any statistical significance. Moreover, Oji et al.[31] reported that the over-expression was observed in most of the patients examined by them, and this high frequency might indicate that the *WT1* gene is one of the critical genetic events in tumorigenesis. They reported that the analysis of the correlation between the expression of *WT1* in colorectal hyperplastic mucosa, adenoma, and carcinoma, is of interest and importance.[31]

Many previous studies[32–34] indicated that the expression of the *PRAME* gene is not significantly associated with relapse-free survival, tumor grade and size, and lymph node status in cases of CRC. These results may be due to

Table 4 Gene expression results

Tumor-associated antigens		Colorectal cancer group ($n = 25$)	Control group ($n = 10$)	*p*-value
		N (%)		
CA9[$]	Positive	24 (96)	1 (10)	< 0.00001
	Negative	1 (4)	9 (90)	
WT1[*]	Positive	11 (44)	1 (10)	0.055
	Negative	14 (56)	9 (90)	
PRAME[#]	Positive	9 (36)	2 (20)	0.357
	Negative	16 (64)	8 (80)	

Notes: [$]Sensitivity: 96%; specificity: 90%; positive predictive value (PPV): 96%; negative predictive value (NPV): 90%; accuracy: 94.29%.
[*]Sensitivity: 44%; specificity: 90%; PPV: 91.67%; NPV: 39.13%; accuracy: 57.14%.
[#]Sensitivity: 36%; specificity: 80%; PPV: 81.82%; NPV: 33.33%; accuracy: 48.57%.

Table 5 Comparison of the expression of tumor-associated antigens of the *CA9*, *WT1*, and *PRAME* genes between the two study groups

Tumor-associated antigens	Colorectal cancer group	Control group	*p*-value
	N (%)		
CA9	24 (96)	1 (4)	< 0.0001
WT1	11 (91.6)	1 (8.4)	< 0.0001
PRAME	9 (81.8)	2 (18.2)	< 0.001

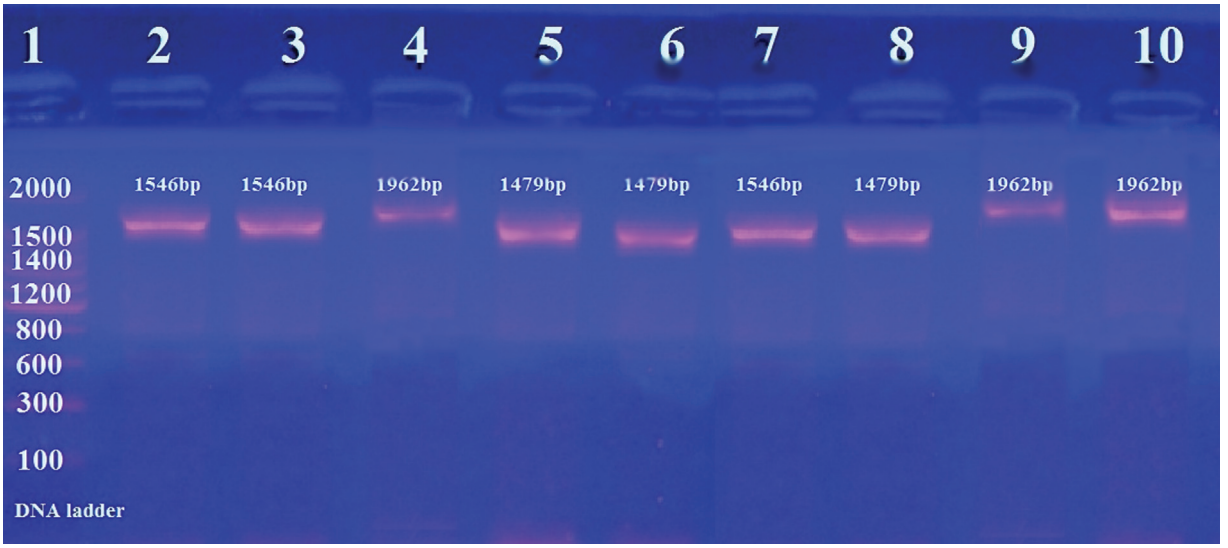

Fig. 2 Gel electrophoresis of amplified DNA from CRC patients by using the primers set *CA9*, *WT1*, and *PRAME* (forward and reversed). Lane 1: DNA ladder. Lanes 2, 3, 7: CRC DNA positive for 1,546 base pairs (bp) (*CA9*). Lanes 4, 9, 10: CRC DNA positive for 1,962 bp (*PRAME*). Lanes 5, 6, 8: CRC DNA positive for 1,479 bp (*WT1*).

the fact that the exact function of the *PRAME* gene in the tumorigenesis of CRC remains controversial.[34]

The analysis of a very small sample of patients (25 patients with CRC and 10 controls) is a main limitation of the present study; further research with larger samples should be considered to confirm the results found.

Conclusion

The overexpression of the *CA9* gene in CRC of high sensitivity and specificity to be used as a tool for discriminating malignant colorectal tumors from benign since with high accuracy compare with the *WT1* and *PRAME* genes. The diagnostic and prognostic indications of *CA9* are more implications to expressed CRC than that of *WT1* and *PRAME*. Furthermore, studies on the molecular mechanisms of the *WT1* and *PRAME* genes are mandatory to provide new information about the function and regulating pathways of these two genes in the tumorigenesis of CRC, since they are expressed, but with low levels of significance. Further research with larger samples should be considered to confirm the results found in the present study. To our knowledge, the present is the first study to report on the importance of the *CA9*, *WT1*, and *PRAME* genes in cases of CRC.

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Conflict of Interests

The authors have no conflict of interests to declare.

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